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(54) Title: OPTIMIZING siRNA BY RNAi ANTISENSE

(57) Abstract: The present invention regards a novel method for identifying potent siRNA sequences that may be used to modify the expression of a target gene sequence. Specifically, the present invention provides a method for the identification of optimal siRNA targeting sites by utilising RNAi antisense. Further, the present invention provides siRNA molecules identified through the present screening method and pharmaceutical preparations comprising said siRNA molecules.

Optimizing siRNA by RNAi antisense

The present invention regards a novel method for identifying potent siRNA sequences that may be used to modify the expression of a target gene sequence. Specifically, the present invention provides a method for the identification of optimal siRNA targeting sites by utilising RNAi antisense. Further, the present invention provides siRNA molecules identified through the present screening method and pharmaceutical preparations comprising said siRNA molecules.

Furthermore, the present invention also provides a method for detecting optimal RNAi antisense molecules which may be used as a therapeutic tool in gene silencing.

BACKGROUND OF THE INVENTION

Antisense effects were in *C. elegans* found both with the antisense and, surprisingly, the sense strand of RNA (1), leading up to the discovery of RNA interference (RNAi) by Andrew Fire and co-workers (2). The potent RNAi process, whereby dsRNA causes specific interference with the expression of homologous endogenous genes, appears to be defence against virus and transposons. This defence has subsequently been shown to exist in a wide range of species (3-5). With the demonstration of the efficacy of short interfering RNAs (siRNA) in human cells (6-8), a valuable tool for both research and therapeutics was created. Now the development have come a full circle with the recent reports that the antisense strand of siRNA (RNAi antisense) is almost as potent as the siRNA duplex (9-11).

The mechanism of action of the RNA interference pathway is still not fully understood and different theories are proposed. Long dsRNA is first processed to shorter 21-23 nt fragments, i.e. siRNA, by an enzyme named Dicer. In the second step the siRNAs produced combine with, and serve as guides for, a ribonuclease complex called RNA-induced silencing complex (RISC), which cleaves the homologous single-stranded mRNAs. However, the siRNA appears to be incorporated into an inactive RISC complex, requiring unwinding of the duplex with concomitant loss of its sense strand for conversion into an active complex (RISC*) (12). RISC cuts the mRNA approximately in the middle of the region paired with the antisense siRNA, after which the mRNA is further degraded.

The RNAi antisense is also incorporated into RISC in HeLa cell extracts and supports RISC-specific target RNA cleavage although at lower efficiency than the siRNA duplex (9, 10). The highly diverging estimates reported for the size of RISC (10, 12, 13), together with the reports of additional RISC-like complexes (14-17) associated with both siRNA and the related microRNAs (18), suggest the existence

of various distinct complexes with possible involvement in different RNA interference pathways.

Since the discovery of RNA interference, and despite of the indistinctness regarding the mechanism of action and the applicability of RNA interference, there has been a perceptible growing interest in identifying siRNA molecules and developing new drugs towards a range of conditions. WO 01/75164 regards RNA sequence-specific mediators of RNA interference and relates to isolated RNA molecules (double stranded; single stranded) of from about 21 -23 nucleotides in general. WO 01/75164 also relates *inter alia* to a method of producing said RNA molecules, e.g. by using the Drosophila *in vitro* system, by chemical synthesis or recombinant techniques. Further, WO 02/44321 disclose *inter alia* isolated double-stranded RNA molecule of 19-25 nucleotides capable of target-specific nucleic acid modifications, a method for processing said RNA molecules and the use thereof.

Recently, great many researchers and pharmaceutical companies have seen the advantage of modulating gene expression by utilizing RNA interference. For example, WO 02/101072 claims methods for modulate the expression of LETM-1 (leucine zipper EF hand transmembrane receptor) by administering a siRNA to a subject. Similarly, WO 02/101072 discloses the modulation of a CD43 encoding nucleic acid by using e.g. siRNA molecules. WO 02/096927 and WO 02078610 disclose methods to affect the expression of vascular endothelial growth factor receptor (VEGF) or PAK2 respectively. Further, WO 02/085289 regards the development of medicaments for the modulation of angiogenesis and thereby identifying siRNA molecules that inhibit the expression of a nucleic acid encoding C1 angiogenesis protein (integrin-linked kinase associated protein, ILKAP)). However, WO 02/101072, WO 02/096927, WO 02078610 and WO 02/085289 do not disclose examples of specific siRNA candidates against the respective target genes.

Also, none of the previous mentioned publications takes into account the fact that even though all gene expression can, in principle, be suppressed by use of e.g. oligonucleotide (synthetic chains), ribozymes or siRNA molecules; there is no reliable way to determine exactly what part of an mRNA sequence is most effectively targeted by siRNA. The position dependence of siRNA efficacy is further supported in the international patent application WO 03/066650, which demonstrates that without revealing any unusual features, siRNA sequences directed towards different sequences of the same mRNA had quite dissimilar efficiency.

Generally, the current apprehension of RNA interference in the prior art, is that finding an effective siRNA is troubled only "occasional ineffectiveness" (21, 22). Also, Thomas Tuschl has previously claimed that RNA interference does not need to be selected for an "optimal" sequence (23). Further, Tuschl and colleagues have

predicted "that it will be possible to design a pair of 21- or 22-nt RNAs to cleave a target RNA at almost any given position" (7).

Contrary to the opinion of Tuschl et al. (7), our results surprisingly show that specific regions of the mRNA is far more efficient in gene silencing and which further demonstrate the necessity for finding optimal siRNA target sequences.

Further, the prevailing results regarding the relative efficiencies of siRNA and RNAi antisense are quite contradictious. Martinez et al. (10) states that RISC activity could not be induced by RNAi antisense in Drosophila lysates, while another research group found that it could (9). Further, Martinez et al. (10) claim as good antisense effects against the protein A/C lamin *in vivo* in HeLa cells as with siRNA. On the other hand, Schwarz et al (9) report that they need eight-fold more antisense to approach the potency of siRNA. Furthermore, the research group of Tuschl has previously alleged that RNAi antisense was not active at all (6). This opinion has later been accredited to lack of 5'phosphorylation of the RNA strand (10). However, the present inventors have shown that prior phosphorylation is not required for *in vivo* activity of RNAi antisense in their assay (11).

Traditionally, chemical modification of nucleic acids has *inter alia* been used to protect single stranded nucleic acid sequences against nuclease degradation and thus obtaining sequences with longer half life. For example, WO 91/15499 discloses 2'O-alkyl oligonucleotides useful as antisense probes. Also, 2-O-methylation has been used to stabilize hammerhead ribozymes (4). However, little is known about the effects of chemical modifications of siRNAs. Further, the presence of large substituents in the 2'hydroxyl of the 5'terminal nucleotide might interfere with the proper phosphorylation of the siRNA shown to be necessary for the activity of the siRNA (12).

Thus, an inherent differential activity in the various siRNAs in a population would mean that different siRNAs would be affected by siRNA modifications, chemical or mutational, in different ways, and generally in a deleterious way, as shown in the exemplary material. Therefore, there is a considerable need for a method to efficiently identify optimal siRNA molecules, which may be chemical modified or not, to be able to develop useful pharmaceutical agents to modulate the expression of a target gene.

There have been proposals for full-genome screenings by utilizing the RNAi pathway in *C.elegans* to e.g. validate potential drug targets, investigate the biological role of validated targets and screen for active compounds (O'Neil et al., (24). Also, EP 0 756 634 B1 disclose a method for the screening of a genetic sequence which is capable of inhibiting, reducing, altering or otherwise modulate the expression of a target nucleotide sequence, e.g. the screening for useful antisense, sense or ribozyme constructs or other nucleic sequences. More

specifically, the method disclosed in EP 0 756 634 B1 makes use of *S. pombe* to evaluate the effect of the introduction of the molecule to be tested on the expression of a target gene in *S. pombe*.

However, the prior art do not disclose a suitable method that may be used to distinguish between efficient and less efficient siRNA candidates that render it possible to develop new useful therapeutic approaches to a variety of disorders. The present invention provides a mean for identifying potent siRNA molecules and RNAi antisense molecules that may be used as the active agent in a pharmaceutical preparation against various diseases and disorders.

SUMMARY OF THE INVENTION

The present inventors have compared RNAi antisense and siRNA using a range of different oligos, demonstrating that the two types of effector molecules behave very similarly with regard to mRNA target position effects, cleavage fragment production, and tolerance for chemical and mutational modifications.

Thus, the present invention provides a method for detecting optimal siRNA targeting sites by targeting a series of sites on an mRNA utilizing RNAi antisense. The preparation of the RNAi to be used and the determination of the optimal siRNA molecules according to the method of the present invention may be provided by various techniques as will be apparent from the description of the invention below. More specifically, in one embodiment, the method according to the present invention comprises the following steps:

a) providing a suitable range of RNAi antisense molecules directed towards the target nucleic acid;

b) introducing each of the RNAi antisense molecules into a cell or a system containing the target nucleic acid;

c) determining the effect of the RNAi antisense molecules on the level of said target nucleic acid and/or expression thereof; and

d) identifying the optimal siRNA molecules from the effects determined in (c).

Preferably, the RNAi antisense molecules are provided by e.g. chemical synthesis, *in vitro* transcription or by endogenous expression directed by a suitable DNA vector.

In one embodiment, the RNAi antisense molecules are introduced into a suitable cell line, more preferably a human keratinocyte cell line HaCaT.

Further, according to one embodiment of the present invention, the RNAi antisense molecules are introduced by cationic liposome transfection, more preferably by Lipofectamin™2000 transfection.

Also, according to other embodiments of the present invention, the RNAi antisense molecules are introduced by e.g. electroporation, microinjection or by coated gold particles shot into cells (GeneGun in plant cells).

In another embodiment of the present invention, the RNAi antisense molecules are introduced by expression from a suitable vector, wherein the vector is introduced according to any of the previous mentioned methods for introducing the RNAi antisense molecules into a cell.

In yet another embodiment of the present invention, the RNAi antisense molecules are introduced into a suitable cell lysate.

In still another embodiment the RNAi antisense molecules are introduced into a suitable *in vitro* expression assay.

Also, according to various embodiments of the present invention, the RNAi antisense efficacy is determined e.g. by Northern blotting analysis, qRT-PCR, Western analysis, primer extension analysis. In still another embodiment of the present invention, the RNAi antisense efficacy is determined by fluorescent marker.

Further, according to still another embodiment the RNAi antisense efficacy is determined by phenotypic marker. More preferably, the phenotypic marker indicates a specific morphological, proliferative or apoptotic characteristics.

In another aspect, the present invention also provides the use of a method according to any of the claims 1 - 22 for the determination of optimal siRNA targeting sites.

Further, the present invention also provides optimal siRNA molecules detected by the method according to the present invention. In one specific embodiment, the present invention provides optimal siRNA molecules which are chemically modified.

In still another aspect, the present invention provides a pharmaceutical preparation comprising one or more siRNA molecules according to any of the claims 23-24, optionally together with pharmaceutical suitable excipients or carriers.

As demonstrated herein, also RNAi antisense molecules may be used as a therapeutic tool in gene silencing. Thus, the present invention regards a method for detecting optimal RNAi antisense molecules. In one embodiment, the method according to the present invention specifically comprises the following steps:

a) providing a suitable range of RNAi antisense molecules directed towards the target nucleic acid;

b) introducing each of the RNAi antisense molecules into a cell or system containing the target nucleic acid;

c) determining the effect of the RNAi antisense molecules on the level of said target nucleic acid and/or expression thereof; and

d) identifying the optimal RNAi antisense molecules from the effects determined in (c).

The RNAi antisense molecules may be provided by the same techniques as mentioned above. Also, the introduction of RNAi antisense molecules and the expression thereof may be performed as mentioned above.

Further, the present invention provides optimal RNAi antisense molecules, chemically modified or not, detected by the method according to the present invention.

Finally, in another aspect, the present invention provides a pharmaceutical preparation comprising one or more RNA antisense molecules according to the present invention, optionally together with pharmaceutically suitable excipients or carriers.

The preparations according to the present invention may comprise any conventional pharmaceutically acceptable adjuvants, carriers, excipients or diluents. Moreover, the preparations according to the present invention may be formulated for parenteral, oral, rectal, mucosal, sublingual or topic administration, for example such as subcutaneous or intramuscular injections liquids or infusion liquids, capsules, tablets, lozenges, granulates, oral suspensions or solutions, or suppositories. Further, the preparations according to the present invention may comprise any suitable amount of the siRNA molecule or RNAi antisense molecule, optionally together with e.g. carriers, adjuvant or excipients in accordance with the appropriate daily dose range. Any medical practitioner or clinician of the art should be able to adapt a suitable dose range for the specific patients.

Additional aspects will be set forth in part in the description, or may be learned by practice of the invention. It is to be understood, however, that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not to be contemplated as restrictive to the scope of the present invention.

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the inventions and thus explains the principles of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. RNAi antisense and siRNA show identical positional effects against same target sites on Tissue Factor mRNA. (A) Dose-response curve. Complexation with Lipofectamine 2000 was performed in one batch for all samples and complexes were diluted in medium immediately before addition to cells. (B) Global and (C) local target position effect. (**B and C**) Northern analysis of Tissue Factor mRNA after transfection with 200 nM RNAi antisense or 100 nM siRNA control. GAPDH was used as a control. Open arrowhead and arrow indicate cleavage product for as-167 and as-372, respectively. Black arrowhead is siRNA control.

Figure 2. Mutational inactivation of as-167. Northern analysis of Tissue Factor mRNA after transfecting with 200 nM RNAi wild type (wt) or single-mutated (as-s3, as-s7, as-s10, as-s13, as-s16: the numerals refer to the position of the mutation, counted from the 5' end of the siRNA sense strand) versions of hTF167i RNAi antisense. Northern analysis of Tissue Factor mRNA after transfecting with 200 nM RNAi antisense or 100 nM siRNA. GAPDH was used as a loading control.

Figure 3. Comparing the influence of chemical modification of RNAi antisense and siRNA. (**A**) Methylational inactivation of as-167. (**B**) Methylational inactivation of hTF167i.

Figure 4. RNAi antisense short-term time-course. Cells were transfected as described and harvested at the indicated time points. TF/GAPDH expression was normalized to levels in mock-transfected cells. The results are representative for two independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described in more detail with reference to the examples. The examples serve only as a representative group of the various embodiments of the present invention. The list of examples is thus not meant to be exclusive.

The term "siRNA" or "siRNA molecule" as used herein means double stranded RNA molecules in which each strand comprises 19-29 nucleotides that may or may not be chemical modified.

The term "RNAi antisense" as used herein means the target-complementary strand of the siRNA, as defined above.

The term "chemical modification of the RNAi antisense", "chemical modified RNAi antisense" , "chemical modification of the siRNA (molecule)" or "chemical modified siRNAi (molecule)" as used herein means any chemical modification of said RNA sequence. Non-limited examples of such chemical modifications are 2'-OH-modification, e.g. alkylation such as methylation; 3' or 5' end modifications such as fluorescent labels, non-standard nucleotides, lipophilic linker molecules or peptides; or modification or exchange of the phosphodiester bond, e.g. with phosporothioates, methylphosphonates, or polyamide.

The term "suitable range of siRNA" as used herein means any set of unique sequences or a mixture thereof, including a randomized collection of such molecules, provided by any of the means previously stated.

There is a commonly held belief that mRNA is generally accessible to siRNA. However, our systematic search (19) demonstrated clear differences in activity of various siRNAs. Also, even if most siRNAs have some activity, some applications may require the identification of the best siRNA. This would be of particular importance for therapeutic applications requiring modifications for example increasing the in vivo stability of siRNA or for delivery of siRNA in vivo. Since such modifications gradually reduce activity (11), only the most effective siRNA would have the necessary excess capacity to tolerate modification and still retain sufficient activity for mRNA targeting.

We have shown that RNAi antisense, although somewhat less active than siRNA, displays an activity that correlates strongly with the position dependent activity of siRNA (**Fig. 1**). This correlation extended to the effects of mutations (**Fig. 2**) and chemical modifications (**Fig. 3**). Despite its lower specific activity, RNAi antisense efficacy still approached that of the corresponding siRNA at moderate and non-toxic concentrations (**Fig. 1A**). These results enable the use of RNAi antisense in screening for optimal siRNA target sites.

Interestingly, we found that RNAi antisense reached maximum activity substantially faster than the corresponding siRNA (**Fig. 4**). This argues that the RNAi antisense enters the RNA interference pathway at a stage closer to the effector nuclease than siRNA. This surprising discovery makes RNAi antisense a potential drug class of its own in contrast to the opinion in the prior art. The possible applications include therapeutic interventions where rapidity of action is of the essence, such as in clinical conditions resulting from acute over-expression of target genes. Our primary target gene, Tissue Factor, is one candidate for such a therapeutic intervention.

An automated search for good target positions would be significantly cheaper and simpler to perform using RNAi antisense instead of siRNA, as there would be no need for synthesis of both strands and annealing them (the latter introducing an

additional step that might be difficult to automate). In the case of chemically synthesized RNAs, automation can be accomplished through streamlining of the following technologies, each of which in itself can be considered off-the-shelf:

1. automated RNA synthesis
2. automated transfection by robotic mixing of RNA, transfection agent and cells
3. automated isolation of total RNA or mRNA (ref QIAGEN)
4. automated determination of target mRNA expression by quantitative real-time RT-PCR (ref QIAGEN or Perkin Elmer)

The screening strategy can also be performed with in vitro transcribed RNA, starting with the synthesis of a DNA strand consisting of the complementary strand of the RNAi antisense in conjunction with the sequence of the minus strand of a phage (T7, T3, Sp6) RNA polymerase promoter. Annealing of the above DNA strand with the sense strand of the promoter would yield a template for in vitro transcription of RNA. Following in vitro transcription (and preferably a purification step), transfection and further analysis would proceed as described for chemically synthesized RNA.

Both chemical synthesis and in vitro transcription can be used to generate either distinct RNA molecules or mixtures of RNA molecules of different complexity. A randomized set of RNAi antisense could also be expressed endogenously from appropriate vectors, preferably of viral origin and from RNA polymerase III promoters. In the above cases, following transfection with the RNA mixture or mixture of encoding DNA vectors, and total RNA isolation, a different analysis step would be required to detect the most effective RNAi antisense molecules. The preferable way to do this is by primer extension analysis, which identifies the most prominent cleavage positions within the target mRNA. From the known sequence of target mRNA, the sequence of RNAi antisense causing the most prominent cleavage events can be inferred.

EXAMPLES

The invention will now be described by way of examples. Although the examples represent preferred embodiments of the present invention (best mode), they are not to be contemplated as restrictive or limiting to the scope of the present invention and the enclosed claims.

MATERIALS AND METHODS

RNAi antisense preparation

21-nucleotide RNAs were synthesized as described (11, 19). Antisense RNA were designated as-N, with N being the corresponding siRNA and mRNA target position (19). The various mutated and chemically modified versions of as-167 were designated as-X, where X is the corresponding siRNA (11).

Cell culture and transfections

The human keratinocyte cell line HaCaT was cultured in serum-free keratinocyte medium (Gibco BRL) supplemented with 2.5 ng/ml epidermal growth factor and 25 µg/ml bovine pituitary extract. The cell line was regularly passaged at sub-confluence and plated one or two days before transfection. HaCaT cells in 6-well plates were transfected at low confluency (<40%) with 1.0 ml 100 nM siRNA in serum-free medium, using Lipofectamine 2000. For complexation, 10 µM stock solution of siRNA or 20 µM stock solution of RNAi antisense was diluted with 10x volume of serum-free medium and mixed with an equal volume of medium-diluted Lipofectamine 2000, at a v/w ratio of liposome to siRNA of 5:2. Batch-dilutions of liposomes were performed for each 6-well plate and allowed to pre-incubate at room temperature for 5-7 minutes before addition to the medium-diluted siRNA. Complexes were replaced with full medium 5 h after initiation of transfection. For standard assays of activity, cells were harvested the day after transfection. For longer incubations and time-course experiments, medium was replaced every second day after transfection.

Northern analysis

Polyadenylated mRNA was isolated using Dynabeads oligo$(dT)_{25}$ (Dynal). Isolated mRNA was fractionated by electrophoresis for 16-18 h on 1.3% agarose/formaldehyde (0.8 M) gels and blotted onto nylon membranes (MagnaCharge, Micron Separations). Membranes were hybridised with random-primed TF (position 61-1217 in cDNA) and GAPDH (1.2 kb) cDNA probes in PerfectHyb hybridisation buffer (Sigma).

RESULTS

RNAi antisense shows lower activity than siRNA

We have previously shown that the maximum depletion of Tissue Factor mRNA by RNAi antisense, at 200 nM concentration, approaches that achieved by the corresponding siRNA (19). As siRNA-mediated inhibition reaches saturation at substantially lower concentrations than the one used for RNAi antisense, the latter might still be substantially less active than siRNA at lower concentrations. Dose-

dependence experiments demonstrated that siRNA was active at approximately 5-6 fold lower concentrations than RNAi antisense (Fig. 1A). Their IC_{50} values were estimated at 5 nM and 30 nM, respectively.

RNAi antisense has target position dependence

A comparison between RNAi antisense and siRNA can, of course, not conclusively prove a shared RNAi pathway, but major deviations between the two could very well decisively falsify our hypothesis of a shared RNAi pathway. We therefore decided to investigate whether RNAi antisense would have the same position effects demonstrated previously for siRNA (19, 20). The efficacies of antisense RNAs targeting sites on TF mRNA coding region from the start-codon (as-77) to the stop-codon (as-929) were evaluated in our quantitative Northern assay (Fig.1B). The results confirmed the position dependence of RNAi antisense efficacy, with the rank order of efficacy correlating strongly with inhibitory data for corresponding siRNA (Pearson's coefficient for N=7 bivariate correlation of inhibition: 0.967, $p<0.001$).

Going on from global position effects to investigate the local area on the mRNA close to the best RNAi antisense, as-167, using overlapping oligos differing only 3 nucleotides from each neighbour, we found that the activity differed sharply with each 3 nucleotide shift (Fig.1C) The rank order also here correlated with the siRNA efficacy order.

RNAi antisense is gradually affected by mutations

We have earlier demonstrated a relatively high tolerance for mutations in an siRNA with high activity (11). These related siRNA, which display a wide range of activities, provided us with a further test of the correspondence between siRNA and RNAi antisense activity. Screening of a set of mutated antisense RNAs demonstrated the same rank order of activity for the antisense candidates as for the corresponding siRNA duplexes (Fig. 2).

RNAi antisense is gradually affected by nucleotide methylation

In a further characterization of the functional anatomy of RNAi antisense, we investigated the effects on activity of gradually increasing degrees of 2'-*O*-methylation, as previously reported for siRNA (11). The mRNA depletion achieved with chemically modified RNAi antisense (Fig. 3A) mirrored the previously observed gradual attenuation of siRNA activity by chemical modification (11) (Fig. 3B). The RNAi antisense with very little modifications (as-M0+2 and as-M1+1) had almost the same activity as the wild type as-167, while more extensive methylation resulted in a gradual decline of activity, that was more pronounced with RNAi antisense. An exception here is M2+2, which lost disproportionally much activity.

This is possibly due to an unfavourable secondary or tertiary structure for incorporation into RISC.

RNAi antisense reaches maximum activity faster than siRNA

In a further comparison of siRNA and RNAi antisense function, we have investigated the time-dependence of the onset of mRNA depletion. Somewhat surprisingly, we found that not only was the RNAi antisense effect stronger than siRNA at 10 h post-transfection, even for the mutationally and chemically modified ones, but the maximum effect of RNAi antisense also seemed to be achieved already at this time-point, as mRNA levels increased somewhat at later time points (Fig. 4). This behaviour extended to the siRNA/RNAi antisense pair targeting hTF372 (data not shown). The faster onset of RNAi antisense-mediated silencing may be due to direct incorporation of single-stranded RNA into the active nuclease complex (RISC*) (12).

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CLAIMS

1. A method for utilizing RNAi antisense for the detection of optimal siRNA targeting sites capable of affecting the level of a target nucleic acid by comprising the targeting of a series of sites on an mRNA,
c h a r a c t e r i z e d in that the method comprises the following steps:

a) providing a suitable range of RNAi antisense molecules directed towards the target nucleic acid;

b) introducing each of the RNAi antisense molecules into a cell or system containing the target nucleic acid;

c) determining the effect of the RNAi antisense molecules on the level of said target nucleic acid determining and/or expression thereof; and
d) identifying the optimal siRNA molecules from the effects determined in (c).

2. Method according to any of the claims 1,
c h a r a c t e r i z e d in that the RNAi antisense molecules are provided by chemical synthesis, *in vitro* transcription or by endogenous expression directed by a suitable DNA vector.

3. Method according to any of the claims 1 -2,
c h a r a c t e r i z e d in that RNAi antisense molecules are introduced into a suitable cell line, preferably the human keratinocyte cell line HaCaT.

4. Method according to any of the claims 3,
c h a r a c t e r i z e d in introducing the RNAi antisense molecules by cationic liposome transfection, Lipofectamin™2000 transfection, electroporation, microinjection, coated gold particles shot into cells (GeneGun in plant cells).

5. Method according to claim 4,
c h a r a c t e r i z e d in that a DNA vector according to claim 3 is introduced according to any of the methods according to claim 5.

6. Method according to any of the claims 1,
c h a r a c t e r i z e d in that RNAi antisense molecules are introduced into a suitable cell lysate or a suitable *in vitro* expression assay.

7. Method according to any of the claims 1,
c h a r a c t e r i z e d in that the RNAi antisense efficacy is determined by

Northern blotting analysis, qRT-PCR, Western analysis, fluorescent marker, primer extension analysis, or phenotypic marker.

8. Method according to claim 7,
c h a r a c t e r i z e d in that the phenotypic marker indicates one or more specific morphological, proliferative or apoptotic characteristics.

9. The use of a method according to any of the claims 1 - 8 for the determination of optimal siRNA targeting sites.

10 siRNA molecules,
c h a r a c t e r i z e d i n that optimal siRNA molecules are detected by the method in any of the claim 1-9

11. siRNA molecule according to claim 10,
c h a r a c t e r i z e d i n that said siRNA molecules are chemically modified.

12. Pharmaceutical preparation,
c h a r a c t e r i z e d i n comprising one or more siRNA molecules according to any of the claims 10-11, optionally together with pharmaceutical suitable excipients and/or carriers.

13. A method for the detection of optimal RNAi antisense targeting sites capable of affecting the level of a target nucleic acid by targeting of a series of sites on an mRNA,
c h a r a c t e r i z e d in that the method comprises the following steps:

a) providing a suitable range of RNAi antisense molecules directed towards the target nucleic acid;

b) introducing each of the RNAi antisense molecules into a cell or a system containing the target nucleic acid;

c) determining the effect of the RNAi antisense molecules on the level of said target nucleic acid and/or expression thereof; and

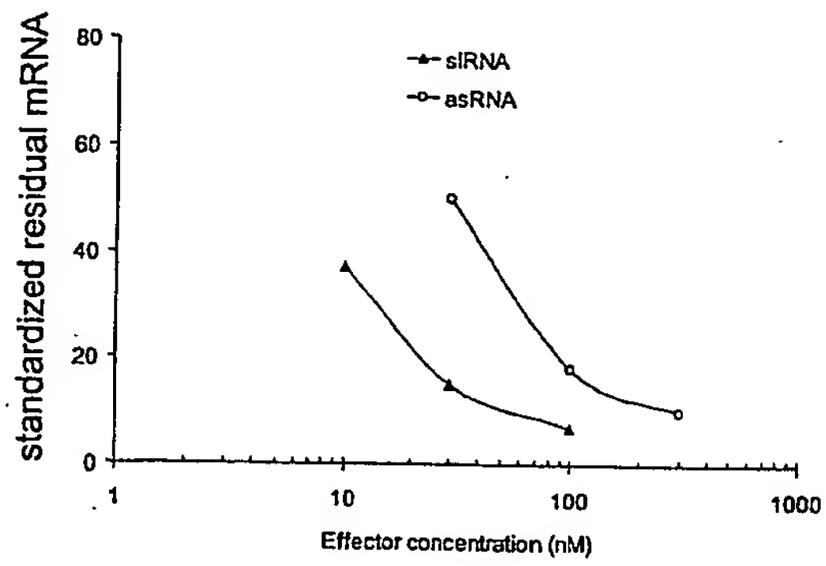
d) identifying the optimal siRNA molecules from the effects determined in (c).

14 RNAi antisense molecule,
c h a r a c t e r i z e d i n that optimal RNAi antisense molecules are detected by the method according to any of the claim 13.

15. RNAi antisense molecule according to claim 14,
c h a r a c t e r i z e d i n that said siRNA molecules are chemically modified.

16. Pharmaceutical preparation,
c h a r a c t e r i z e d i n comprising one or more RNA antisense molecules according to any of the claims 13, optionally together with pharmaceutical suitable excipients and/or carriers.

A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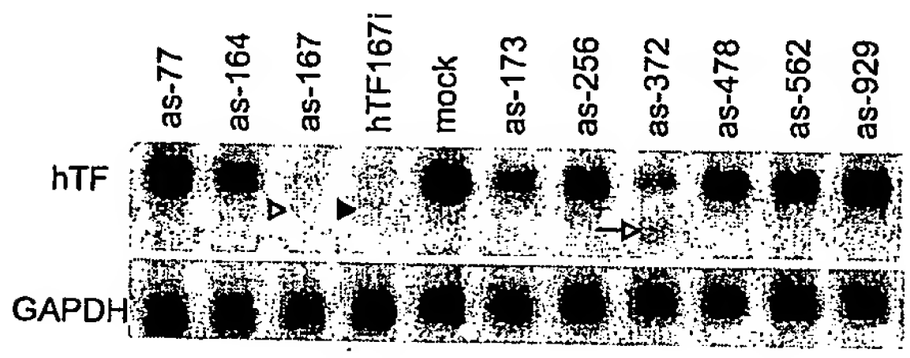
80
60
40
20
0
siRNA
asRNA
1
10
100
1000
Effector concentration (nM)

B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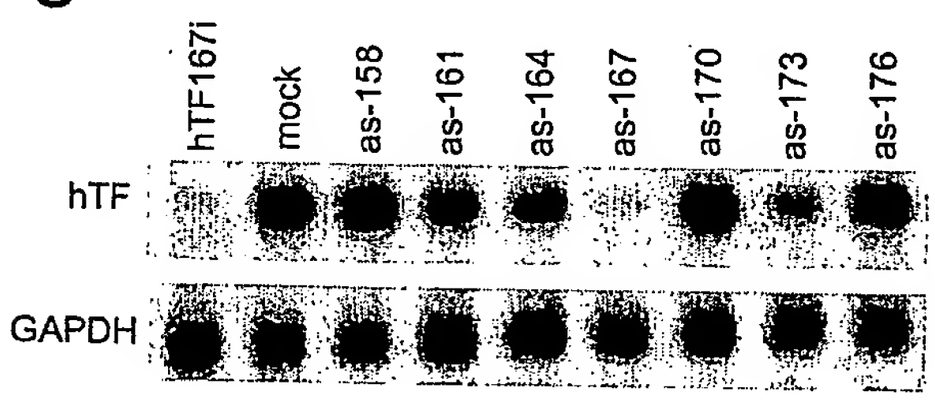
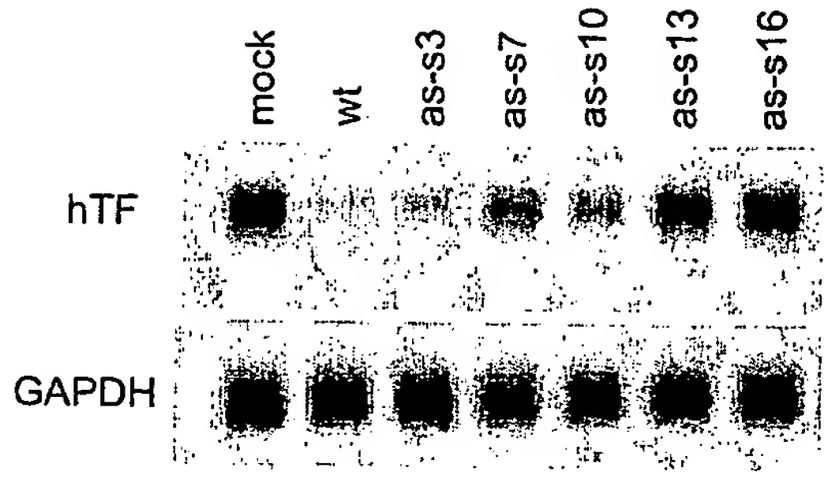
as-164
as-167
hTF167i
mock
as-173
as-256
as-372
as-478
as-562
as-929
hTF
GAPDH

C

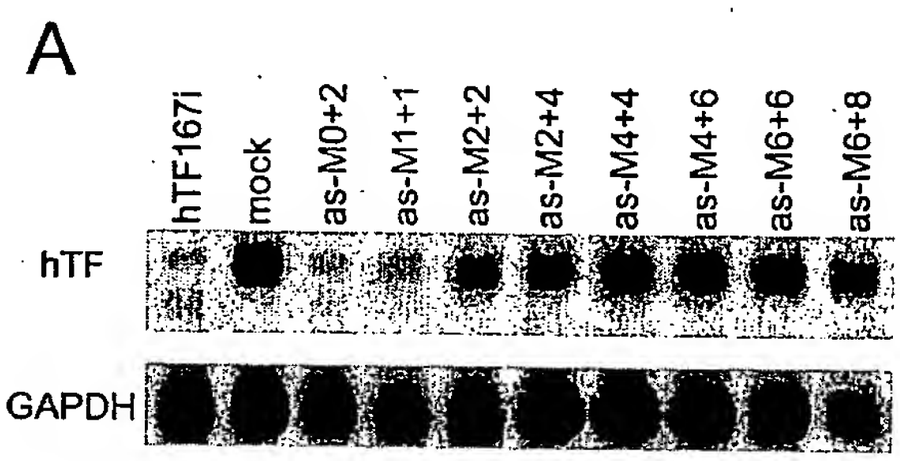

hTF167i
mock
as-158
as-161
as-164
as-167
as-170
as-173
as-176
hTF
GAPDH

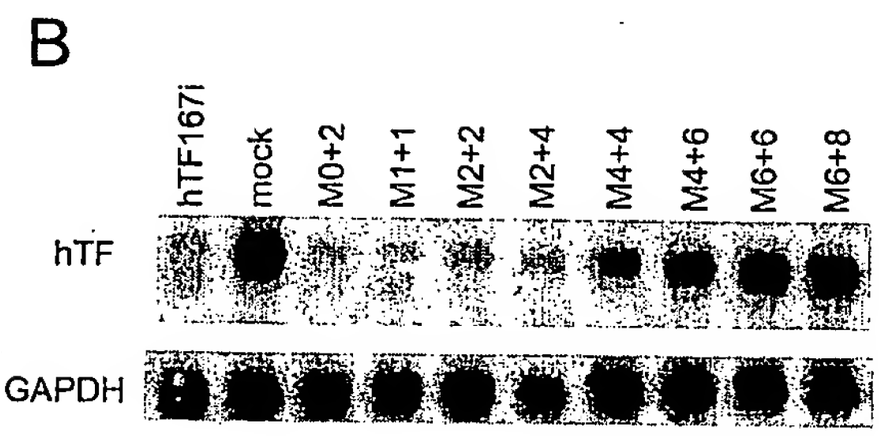
(Fig. 1)

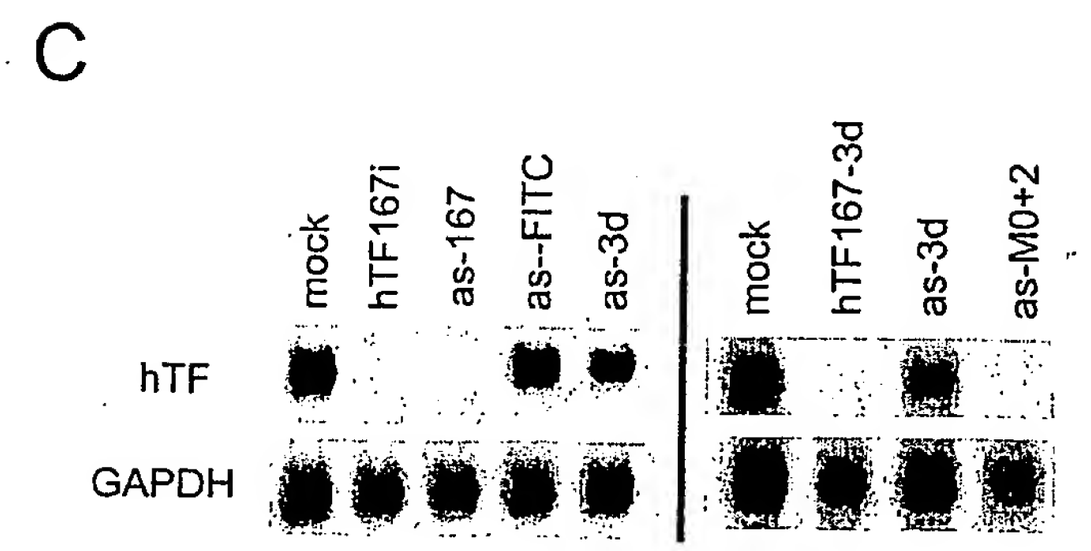
Figur 2



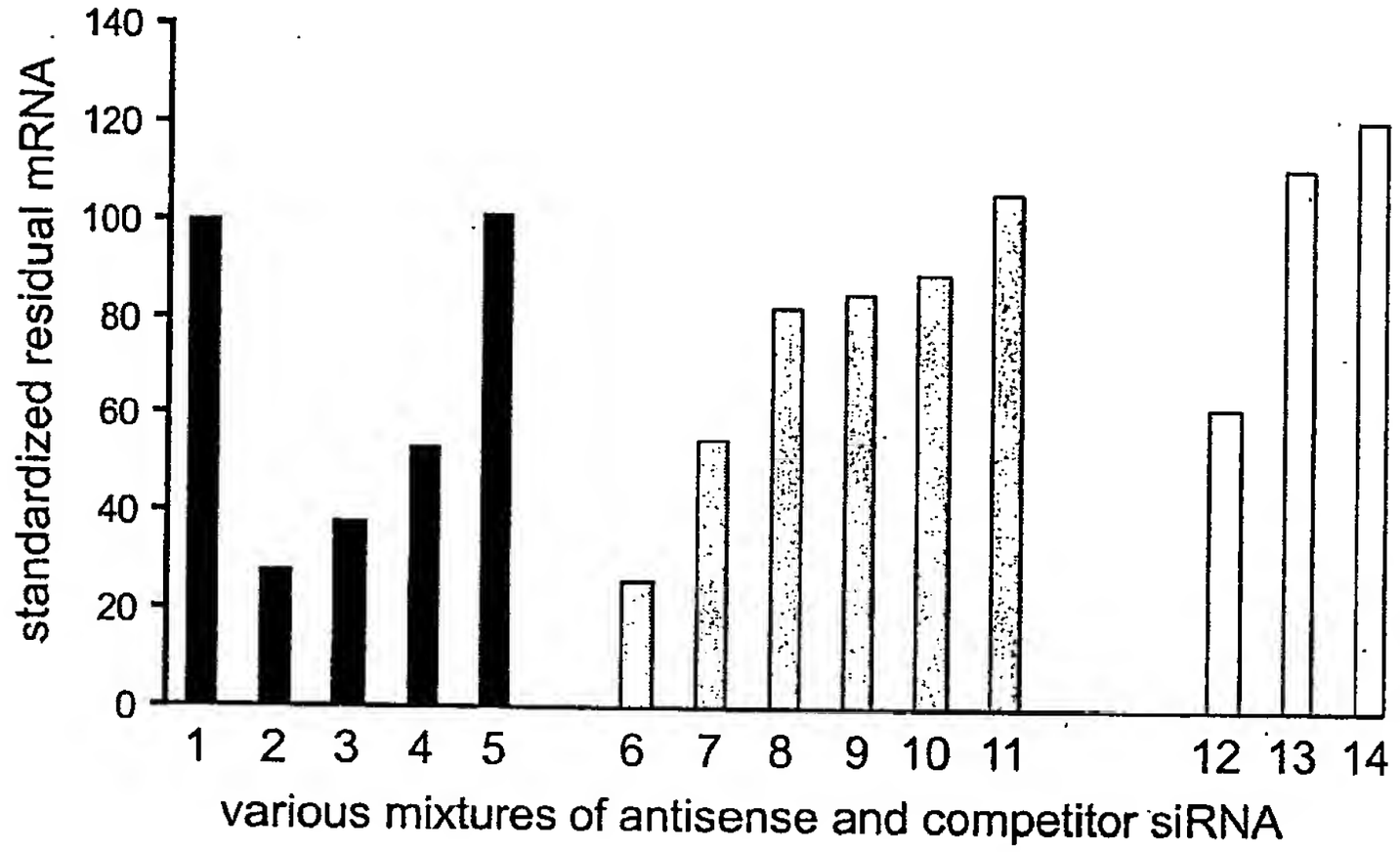
Figur 3







Figur 4


standardized residual mRNA
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
various mixtures of antisense and competitor siRNA

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO2004/000007

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12Q1/68 A61K31/713

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VICKERS TIMOTHY A ET AL: "Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents: A comparative analysis." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 9, 28 February 2003 (2003-02-28), pages 7108-7118, XP002283905 ISSN: 0021-9258 *** PUBLISHED ONLINE on 23.12.2002 **** page 7110, left-hand column, last paragraph page 7113, left-hand column -right-hand column, paragraph 1 --- -/--	1,2,4,6, 7,9,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 2004	08/07/2004
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Andres, S

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO2004/000007

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHWARZ D S ET AL: "EVIDENCE THAT SIRNAS FUNCTION AS GUIDES, NOT PRIMERS, IN THE DROSOPHILA AND HUMAN RNAI PATHWAYS" MOLECULAR CELL, vol. 10, no. 3, September 2002 (2002-09), pages 537-548, XP009019083 ISSN: 1097-2765 cited in the application page 539, right-hand column, last paragraph -page 541 ---	1-9
X	WO 02/090590 A (BAKER BRENDA F ; FREIER SUSAN M (US); ISIS PHARMACEUTICALS INC (US)) 14 November 2002 (2002-11-14)	13
Y	page 85; example 15 page 77; example 10 ---	1-9
X	SOHAIL M ET AL: "OLIGONUCLEOTIDE SCANNING ARRAYS: APPLICATION TO HIGH-THROUGHPUT SCREENING FOR EFFECTIVE ANTISENSE REAGENTS AND THE STUDY OF NUCLEIC ACID INTERACTIONS" ADVANCES IN BIOCHEMICAL ENGINEERING, BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 77, 2002, pages 43-56, XP008026711 ISSN: 0724-6145 the whole document ---	13
X	US 5 525 468 A (MCSWIGGEN JAMES A) 11 June 1996 (1996-06-11) column 6, line 49 -column 7, line 60 claims ---	13
A	HOLEN T ET AL: "Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor" NUCLEIC ACIDS RESEARCH, vol. 30, no. 8, 15 April 2002 (2002-04-15), pages 1757-1766, XP002232890 ISSN: 0305-1048 cited in the application the whole document ---	3
A	ELBASHIR SAYDA M ET AL: "RNA interference is mediated by 21- and 22-nucleotide RNAs" GENES AND DEVELOPMENT, vol. 15, no. 2, 15 January 2001 (2001-01-15), pages 188-200, XP002204651 ISSN: 0890-9369 cited in the application ---	

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO2004/000007

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	AMARZGUIOUI M ET AL: "Tolerance for mutations and chemical modifications in a siRNA" NUCLEIC ACIDS RESEARCH, vol. 31, no. 2, 15 January 2003 (2003-01-15), pages 589-595, XP002270887 ISSN: 0305-1048 cited in the application page 592, left-hand column -page 593, left-hand column ---	1-4,6-9
P,X	WO 03/100093 A (MACAULAY VALENTINE MOYA ; ISIS INNOVATION (GB); SOHAIL MUHAMMAD (GB)) 4 December 2003 (2003-12-04) page 2, line 22 - last line page 6, line 4 -page 10 page 27 -page 35; examples 2,3 claims ---	1-7,9
T	HOLEN TORGEIR ET AL: "Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway." NUCLEIC ACIDS RESEARCH, vol. 31, no. 9, 1 May 2003 (2003-05-01), pages 2401-2407, XP002283906 ISSN: 0305-1048 the whole document -----	

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/NO2004/000007

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1 and 2 encompass methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 10-12,14-16
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 10-12,14-16

Present claims 10-12 and 14-16 relate to products defined soleley by reference to the method by which they have been produced. The claims cover all products having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for any of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the products by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search impossible. Consequently, NO search has been carried out for these claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NO2004/000007

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 02090590	A	14-11-2002	US	6399379 B1	04-06-2002
			EP	1390547 A1	25-02-2004
			WO	02090590 A1	14-11-2002
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			AU	687736 B2	05-03-1998
			CA	2135646 A1	25-11-1993
			EP	1288296 A2	05-03-2003
			EP	0642589 A1	15-03-1995
			JP	8500481 T	23-01-1996
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			JP	2000342286 A	12-12-2000
			WO	9323569 A1	25-11-1993
WO 03100093	A	04-12-2003	WO	03100093 A2	04-12-2003